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# *Bacillus thuringiensis* RESISTANCE MONITORING PROGRAM FOR TOBACCO BUDWORM AND BOLLWORM IN 2003

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## Abstract

The susceptibility of the tobacco budworm (*Heliothis virescens* [F.]) and bollworm *(Helicoverpa zea* [Boddie]) populations from 14 cotton-producing states to Cry1Ac protein of *Bacillus thuringiensis* was monitored for the eighth consecutive year in

2003. The survivorship of first generation larvae obtained from mass mating males captured in pheromone traps near cotton fields (wild population) with laboratory-adapted females (susceptible strain), was determined at 2 diagnostic concentrations for each species plus an untreated control. Survivorship of those larvae was compared to survivorship of the laboratory strain. Survival of the 40 strains of *Heliothis virescens* and 97 strains of *Helicoverpa zea* tested between April and October, 2003 was not elevated above that in the susceptible strain using current methodology. However, the current method does have limitations, and additions and modifications to that methodology are discussed.

## Introduction

Controversy about insect resistance management (IRM) has arisen since the deployment of transgenic cotton, but at the grower level, acceptance of this technology has been rapid constituting an example of how these plants can transform the agricultural landscape. In the U.S. currently, transgenic cotton represents 73% of the planted area (Aldhous 2003) Because transgenic cotton constantly expresses the Cry1Ac protein from *Bacillus thuringiensis* Berliner (Bt), and the widespread and prolonged exposure to Bt proteins provides a constant selection pressure, representing one of the largest selections for resistance development in insect populations the world has ever seen (Tabashnik et al. 2003). In the U.S., an IRM strategy for Bt cotton was mandated by the Environmental Protection Agency that is based on the premise that the transgenic plants express a "high dose" of the protein and implementation of a structured refuge will mitigate the likelihood of resistance evolution (Environmental Protection Agency 2001). This strategy is believed to have helped maintain the susceptibility of target pests such as tobacco budworm and pink bollworm (*Pectinophora gossypiella* [Sauders]) to the Cry1Ac protein in current commercial varieties (BollGard®). The detection of resistance development to transgenic cotton plants expressing *Bacillus thuringiensis* toxins is an important consideration for the preservation of this technology. Since 1996, a program has been conducted yearly in the major cotton areas of the U.S. to monitor resistance of target insects to the *Bacillus thuringiensis* Cry1Ac protein in transgenic cotton plants. This program, which has been continuously expanded and improved, now covers 14 states, and involves more than 30 researchers who contribute important information to industry and the US Environmental Protection Agency. Results from this program in 2003 are included in this report.

## Materials and Methods

For the Beltwide monitoring program, male bollworms (*Helicoverpa zea* [Boddie]) and tobacco budworms were captured in pheromone traps near cotton fields throughout the U.S. cotton region (Alabama, Arkansas, Florida, Georgia, Louisiana, Mississippi, New Mexico, North Carolina, Oklahoma, South Carolina, Tennessee, Texas, and Virginia) (Table 1) and shipped overnight to the Southern Insect Management Research Unit, of the USDA Agricultural Research Service in Stoneville, MS in pint-size cardboard containers ($\leq$ 40 males / container). Those moths were mass-mated with laboratory reared females (Cry1Ac susceptible colony) in carton buckets containing $\leq$ 40 males and 40 females each. Moths were fed 10% sugar solution and maintained at 28 ± 2 ºC , 65 ± 5% RH, under 14:10 h L:D luminosity. Females, in general, laid eggs on a piece of cheese cloth on top of each bucket. Egg sheets were replaced daily. On the second day of high oviposition, generally the 4$^{th}$ day after placing males and females together, eggs were washed from the cloths, dried on filter paper and set to hatch inside a 472-ml clear plastic container in an incubator under the same environmental conditions as previously described. Cry1Ac protein, obtained from MVP II® insecticide, was incorporated into Bio-Serv® diet at 0.05 and 0.1 µg of Cry1Ac per ml of diet for tobacco budworm and 100 and 250 µg per ml for bollworm. Neonates were placed on each treatment in individual 30-ml diet cups containing either 5 ml of Cry1Ac treated or 10 ml of control diet. In addition, neonates from a susceptible colony were placed on diet with each concentration. Larvae were kept in a room with controlled environmental conditions as previously described. Mortality, assessed as larvae not moving after probed, was recorded 7 days later. Data presented in this report, except for Table 5, have not been transformed.

### Additions and Modifications Made in 2003

1) To lower the mortality occurring on control diet, the survivorship and development of tobacco budworms and bollworms was compared between 4 replications of 15 30-ml cups containing either 5 ml or 10 ml of diet and 4 replications of 16 1-ml micro-wells. These tests were repeated 3 times for the first and 4 times for the second mentioned insect. Larval mortality and weight was recorded 7 days later.

2) The rating mortality was evaluated by comparing values obtained considering larval "death," if it was not able to move after being probed, versus larvae "weighing equal to or less than" the original neonate weight (new method). For these tests (10 performed on tobacco budworm and 4 on bollworm), 4 replications of 15 30-ml cups or 4 replications of 16 1-ml micro-wells were setup with each diagnostic concentration. Mortality was assessed 7 days after inoculation.

3) One extra diagnostic concentration was incorporated into the program for each species following discussions with key stakeholders of this program. The new diagnostic concentrations were 0.1 µg of Cry1Ac / ml of diet for *H. virescens* and 100 µg / ml for *H. zea*. Rates utilized in the past (0.05 µg for TBW and 250 µg for BW) were retained throughout the entire season. The goal of including these concentrations was to test an intermediate value for BW and a higher one for TBW that

perhaps more accurately discriminate resistant individuals. New concentrations may be further adjusted in 2004 to provide a more accurate discrimination of "tolerant" individuals.

## Results and Discussion

Based on results of the experiments of different containers [1)], testing was conducted on 30-ml cups instead of the previously used 16-microwell plates. This change was implemented in June 2003 and lasted for the duration of the testing season. Mortality on non-treated diet was significantly reduced in 30-ml cups compared to 16-microwell plates (Table 2). This change decreased confinement of larvae and simplified preparation and larval inoculation for the laboratory personnel.

In the mortality rating experiment [2)], mortality values were higher for both diagnostic concentrations using larval weight as a measure of survivorship, while percent mortality on non-treated diet was not affected (Table 3). The impact was less apparent with tobacco budworm than with bollworm. This is probably a result of differences in the relative susceptibility of these species to *B. thuringiensis* (Luttrell et al. 1999). Individually weighing each surviving larvae is a time-consuming task, but results obtained from this methodology will increase our knowledge of the performance of these insects exposed to Bt-treated diet.

In 2003, 40 strains of tobacco budworm and 97 strains of bollworm were tested. The majority of the strains (100% tobacco budworm and 94.8% bollworm) did not demonstrate increased tolerance to Cry1Ac compared with the susceptible colony. All data not included, only averages (Table 4). However, there were 5 strains of bollworm (1 from FL, 2 from LA, and 2 from VA) that exhibited elevated tolerance (>10%) to at least one diagnostic concentration. Mortality on Cry1Ac treated diet ranged from 10.8% to 65.5% on the 100 µg / ml concentration and 6.4% to 41.8% on the 250 µg / ml concentration. Mortality of these strains on each treatment was corrected for mortality on the non-treated diet using Abbott's formula (Abbott 1925). Corrected mortality in those strains was compared to corrected mortality in the susceptible strain using a two sample paired t-test. Differences between field strains and the laboratory strain were not significantly different ($P>0.05$) (Table 5). Small changes in tolerance to Cry1Ac in bollworm have been already documented with the data generated from this program (Hardee et al. 2001). The limitation of utilizing this methodology restricted us to detect resistance to Cry1Ac only if the resistance trait in males coming from the field is dominant or sex-linked. Additional changes to the program will address these issues in the future.

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## Disclaimer

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Mention of trade names or commercial products in this report is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture, or by any of the educational institutions.

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Table 1. Counties / Parishes represented by State in the *Bacillus thuringiensis* resistance monitoring program for 2003.

| STATE | COUNTY / PARISH |
|---|---|
| ALABAMA | Henry. |
| ARKANSAS | Ashley, Desha, Drew, Little River, and Mississippi. |
| FLORIDA | Gadsden. |
| GEORGIA | Berriem, Burke, Colquitt, Irwin, Montgomery, Tift, Turner and Sumter. |
| LOUISIANA | Catahoula, Franklin, Rapides, Richland, and Tensas. |
| MISSISSIPPI | Calhoun, Lee, Monroe, Noxabee, and Washington. |
| NORTH CAROLINA | Washington. |
| NEW MEXICO | Curry. |
| OKLAHOMA | Harmon and Jackson. |
| SOUTH CAROLINA | Darlington, Dillon, Florence, Lee, Marion, Marlboro, Mitchell, and Sumter. |
| TENNESSEE | Madison |
| TEXAS | Burleson, Lubbock, and Nueces. |
| VIRGINIA | Accomack, Essex, King and Queen, New Kent, Northampton, Prince George, Southampton, and Suffolk. |

Table 2. Percent control mortality and larval weight 7 days after the initiation of the bioassay comparing neonates confined in 30-ml diet cups[1] and micro-wells[2].

| 30-ml cups with 5 ml of diet | | 30-ml cups with 10 ml of diet | | Micro-wells with 1 ml of diet | |
|---|---|---|---|---|---|
| % Mortality | Larval weight (g) | % Mortality | Larval weight (g) | % Mortality | Larval weight (g) |
| ***Helicoverpa zea*[3]** | | | | | |
| 7.8 | 0.0522 | 9.9 | 0.1033 | 33.1 | 0.0633 |
| ***Heliothis virescens*** | | | | | |
| 6.6 | 0.0930 | 10.0 | 0.1237 | 7.8 | 0.1486 |

[1]Solo® cups.
[2]C-D International, Inc.
[3]Average of 4 tests.

Table 3. Differences in percent mortality obtained utilizing 2 assessment criteria: 1) inspecting larvae for mobility (larva moves after probing) and 2) failure to gain weight (≤ to the initial neonate weight).

| ***Helicoverpa zea* Percent Mortality[1]** | | | | | |
|---|---|---|---|---|---|
| Larva moves | | | Larva does not gain weight | | |
| Control diet | 100 µg / ml | 250 µg / ml | Control diet | 100 µg / ml | 250 µg / ml |
| 5.3 % | 43.9 % | 67.3 % | 5.3 % | 68.3 % | 90.0 % |
| ***Heliothis virescens* Percent Mortality[2]** | | | | | |
| Control diet | 0.05 µg / ml | 0.1 µg / ml | Control diet | 0.05 µg / ml | 0.1 µg / Ml |
| 19.7 % | 42.1 % | 40.5 % | 20.2 % | 45.9 % | 45.2 % |

[1]Average of 4 tests.
[2]Average of 10 tests.

Table 4. Average mortality of *Helicoverpa zea* and *Heliothis virescens* larvae exposed to different diagnostic concentrations containing *Bacillus thuringiensis* Cry1Ac protein for 7 days.

| | **Field strain[1]** | | | **Susceptible colony** | | |
|---|---|---|---|---|---|---|
| | ***Helicoverpa zea*** | | | | | |
| **Location (number of tests)** | **0 µg** | **100 µg** | **250 µg** | **0 µg** | **100 µg** | **250 µg** |
| ALABAMA (1) | 0 | 80.0 | 73.0 | 6.3 | 81.8 | 86.3 |
| ARKANSAS (14) | 9.8 | 73.2 | 79.6 | 4.6 | 72.2 | 77.8 |
| FLORIDA (3) | 7.0 | 61.0 | 66.0 | 4.0 | 72.0 | 79.0 |
| GEORGIA (1) | 8.0 | 90.0 | 93.0 | 1.0 | 92.0 | 93.0 |
| LOUISIANA (4) | 4.0 | 67.0 | 90.0 | 6.0 | 92.5 | 96.0 |
| MISSISSIPPI (20) | 5.3 | 86.3 | 88.3 | 5.3 | 85.5 | 89.1 |
| NORTH CAROLINA (3) | 16.0 | 79.0 | 88.0 | 6.0 | 80.0 | 88.0 |
| NEW MEXICO (2) | 3.0 | 80.0 | 97.0 | 6.0 | 92.0 | 93.0 |
| OKLAHOMA (8) | 5.0 | 79.0 | 80.0 | 8.0 | 79.0 | 84.0 |
| SOUTH CAROLINA (2) | 1.0 | 74.0 | 74.0 | 12.0 | 79.0 | 78.0 |
| TENNESSEE (5) | 8.0 | 86.0 | 88.0 | 9.0 | 80.0 | 88.0 |
| TEXAS (27) | 13.0 | 71.0 | 74.0 | 9.0 | 80.0 | 84.0 |
| VIRGINIA (7) | 7.0 | 68.0 | 74.0 | 5.0 | 78.0 | 86.0 |
| | ***Heliothis virescens*** | | | | | |
| | 0 µg | 0.05 µg | 0.1 µg | 0 µg | 0.05 µg | 0.1 µg |
| ALABAMA (2) | 15.0 | 35.0 | 38.0 | 3.0 | 12.0 | 18.0 |
| ARKANSAS (4) | 14.5 | 37.0 | 39.5 | 16.5 | 44.5 | 58.0 |
| FLORIDA (1) | 0 | 7.0 | 20.0 | 3.0 | 12.0 | 18.0 |
| MISSISSIPPI (10) | 20.3 | 63.0 | 62.2 | 7.0 | 42.9 | 51.6 |
| TEXAS (22) | 14.0 | 42.0 | 52.5 | 12.5 | 46.0 | 52.5 |

[1]F1 progeny obtained by crossing wild males captured in pheromone traps with Cry1Ac susceptible females reared under laboratory conditions.

Table 5. Analysis (Two sample paired t-test) of *Helicoverpa zea* mortality of field[1] strains that demonstrated elevated survivorship to 2 *Bacillus thuringiensis* Cry1Ac protein diagnostic concentrations compared with a laboratory (susceptible) strain.

| | **100 µg / ml** | | **250 µg / ml** | |
|---|---|---|---|---|
| **Location and date of cross** | **Field** | **Susceptible** | **Field** | **Susceptible** |
| Florida (21Aug03) | 41.7 a<br>*P*=0.8892 | 50.8 a | 48.4 a<br>*P*=0.9857 | 64.1 a |
| Louisiana (17Jul03) | 58.8 a<br>*P*= 0. 9654 | 92.5 a | 83.8 a<br>*P*= 0.8911 | 98.2 a |
| Louisiana (20Jul03) | 71.6 a<br>*P*= 0.9315 | 91.4 a | 86.6 a<br>*P*= 0.8732 | 93.3 a |
| Virginia (22Jul03) | 81.2 a<br>*P*= 0.7880 | 91.4 a | 70.6 a<br>*P*= 0.9829 | 93.3 a |
| Virginia (05Sep03) | 56.6 a<br>*P*= 0.9901 | 89.5 a | 74.9 a<br>*P*= 0.9868 | 98.2 a |

[1]First generation obtained from mass mating males obtained from pheromone traps near cotton fields with laboratory adapted (susceptible) females.
Means in a row by treatment followed by the same letter are not significantly different (*P*= 0.05).